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Alcaligenes eutrophus CH34 Is a Facultative Chemolithotroph with Plasmid-Bound Resistance to Heavy Metals

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***Alcaligenes eutrophus* strain CH34, which was isolated as a bacterium resistant to cobalt, zinc, and cadmium ions, shares with *A. eutrophus* strain H16 the ability to grow lithoautotrophically on molecular hydrogen, to form a cytoplasmic NAD-reducing and a membrane-bound hydrogenase, and most metabolic attributes; however, it does not grow on fructose. Strain CH34 contains two plasmids, pMOL28 (163 kilobases) specifying nickel, mercury, and cobalt resistance and pMOL30 (238 kilobases) specifying zinc, cadmium, mercury, and cobalt resistance. The plasmids are self-transmissible in homologous matings, but at low frequencies. The transfer frequency was strongly increased with IncP1 plasmids RP4 and pUZ8 as helper plasmids. The phenotypes of the wild type, cured strains, and transconjugants are characterized by the following MICs (Micromolar) in strains with the indicated phenotypes: Nic^+, 2.5; Nic^-, 0.6; Cob^+A, 5.0; Cob^+B, 20.0; Cob^-, < 0.07; Zin^+, 12.0; Zin^-, 0.6; Cad^+, 2.5; and Cad^-, 0.6. Plasmid-free cells of strain CH34 are still able to grow lithoautotrophically and to form both hydrogenases, indicating that the hydrogenase genes are located on the chromosome, in contrast to the Hox structural genes of strain H16, which are located on the megaplasmid pHG1 (450 kilobases).**

The physiological group of the hydrogen-oxidizing bacteria is taxonomically heterogeneous and comprises at least 25 species among 9 genera (4, 9). With respect to the key enzymes of lithotrophy, the hydrogenases, three groups are differentiated. The majority of the hydrogen-oxidizing bacteria contains a membrane-bound hydrogenase only. The second group comprises a few species of the genus *Alcaligenes* (*A. eutrophus*, *A. ruhlandii*, *A. hydrogenophilus*); they are characterized by the ability to form two hydrogenases, a cytoplasmic, NAD-reducing hydrogenase and a membrane-bound hydrogenase. The third group is represented by *Nocardia opaca*, containing only a cytoplasmic NAD-reducing enzyme.

The detection of a large conjugative plasmid of a molecular size of about 450 kilobases (kb) in *A. eutrophus* and its transfer to Hox^- cells lacking the plasmid indicated that the ability to oxidize hydrogen in *A. eutrophus* is encoded by a plasmid (3, 14). Recently, another strain of hydrogen-oxidizing bacteria was described which is unusual owing to its resistance to Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} ions (26). Preliminary experiments indicated that this strain CH34 grows similarly well as *A. eutrophus* and shares the ability to form the two types of hydrogenase.

The present study was aimed at characterizing the new strain CH34 of *A. eutrophus* with respect to its growth properties, hydrogenase activities, susceptibility to growth inhibition by metal ions, respective mutants by plasmid analysis, and genetic transfer of its native plasmids.

MATERIALS AND METHODS

Organisms. *A. eutrophus* CH34 (DSM2839) was isolated from a decantation tank of a zinc factory (25, 26) and was kept on various media. The mutant derivatives of this strain and further bacteria used in this study are listed in Table 1.

Growth media. For measuring growth rates, substrate utilization, and enzyme activities the cells were grown in the mineral salts medium of Schlegel et al. (33). For heterotrophic growth the medium was supplemented by organic carbon sources (0.2 to 1.0%, wt/vol), and cells were grown under air. For lithoautotrophic growth organic compounds were omitted from the medium, and the gas atmosphere contained a mixture of H_2, O_2, and CO_2 (8:1:1; vol/vol). Growth rates were determined by optical density measurements of suspensions growing in 30 ml of Tris medium–0.2% sodium gluconate contained in 300-ml Erlenmeyer flasks, which were shaken in a water bath at 30°C.

Determination of MIC values. For testing the degree of resistance to nickel, cobalt, cadmium, and zinc ions the following four media were compared; (i) CV medium contained 5 g of yeast extract (Difco Laboratories), 5 g of Casamino Acids (Difco), and 2 g of sodium gluconate in 1,000 ml of distilled water (29); (ii) TY medium contained 10 g of Bacto-tryptone (Difco), 5 g of yeast extract, 5 g of NaCl, and 2 g of sodium gluconate in 1,000 ml of water, (iii) Tris medium (12) contained 6.06 g of Tris, 4.68 g of NaCl, 1.49 g of KCl, 1.07 g of NH_4Cl, 0.43 g of Na_2SO_4, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.03 g of $CaCl_2 \cdot 2H_2O$, 0.23 g of $Na_2HPO_4 \cdot 12H_2O$, 0.005 g of Fe(III)(NH_4) citrate, and 1 ml of the trace element solution SL 7 of Biebl and Pfennig (7) in 1,000 ml of distilled water; (iv) mineral salt medium (33). Solidified media contained 15 g of agar per liter. Analytical grade salts $CdCl_2 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, and $ZnCl_2$ (E. Merck AG, Darmstadt, Federal Republic of Germany) were used to prepare 1.0 M stock solutions. They were sterilized by autoclaving.

For the determination of the MIC (the lowest concentration of metal salts at which no CFU were observed), Tris-gluconate-agar plates containing 20 to 0.078 mM ion concentrations (in 2:1 dilution steps) were used for cadmium, cobalt, zinc, and nickel. For mercury (added as merbromine or mercuric chloride), TY agar was used. Col-

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TABLE 1. Bacterial strains

Strain	Resistance to heavy metals[a]					Other relevant markers	Plasmids	Origin or reference
	Ni	CoA	CoB	Cd	Zn			
A. eutrophus CH34 derivatives								
CH34 (wild type)	r		r	r	r	Aut$^+$, prototrophic	pMOL28, pMOL30	Mergeay et al. (26)
AE128	s		r	r	r	Aut$^+$, prototrophic	pMOL30	This study
AE126	r	r	s	s	s	Aut$^+$, prototrophic	pMOL28	This study
AE104	s	s	s	s	s	Aut$^+$, prototrophic	None	This study
AE43 (MO3)	s		r	r	r		pMOL30, pHG13c	Gerstenberg et al. (17)
AE81	r		r	r	r	*trpE50 leu-27 met-81*	pMOL28, pMOL30	Lejeune et al. (22)
AE136	r		r	r	r	*trpE50 leu-27 met-81* Km^r Ap^r Tc^r	pMOL28, pMOL30 RP4	Transconjugant of AE81 × CM140
AE138	r		r	r	r	*trpE50 leu-27 met-81* Km^r Tc^r Hg^r Km^r, Tc^r, Hg^r	pMOL28, pMOL30, pUZ8 RP4, pUZ8	Transconjugant of AE81 × CM237
AE110	s	s	s	s	s	Nal.110	None	This study, from AE104
AE176	r	r	s	s	s	Lys.176	pMOL28	This study, from AE126
AE194	s		r	r	r	Lys.194	pMOL30	This study, from AE128
Escherichia coli CM140	s	s	s	s	s	*pro met*	RP4	C. I. Kado
E. coli CM237	s	s	s	s	s	*pro met* Rif^r	pUZ8	J. P. Hernalsteens
A. eutrophus H16 (DSM 428, ATCC 17699)	s	s	s	s	s	Aut$^+$, prototrophic	pHG1	Friedrich et al. (15)
Rhizobium meliloti MVII							pRMVII, 1, 2, 3, and 4	A. Pühler

[a] r, Resistant; s, sensitive.

onies were counted after 3 days (TY plates with mercury compounds) or 5 days (Tris plates).

The following concentrations of metal were chosen with Tris medium for the differentiation between metal-resistant and -sensitive strains or mutants of *A. eutrophus*: nickel, 0.6 to 1 mM; cobalt, 1 and 5 mM; cadmium, 0.8 to 1 mM; zinc, 1 to 2.5 mM. The results were not influenced by the method of adding the metal salts either to the liquid agar before pouring or onto the solidified agar. For testing heavy metal resistance on TY agar the following concentrations were used: nickel, 3 mM; cobalt, 5 mM; cadmium, 3 mM; zinc, 10 mM; mercury, 0.5 mM. These concentrations gave the same phenotypic responses as those on Tris-gluconate agar.

Plasmid curing. Erlenmeyer flasks (50 ml) containing 5 ml of Tris-gluconate medium and various concentrations of mitomycin C, ethidium bromide, novobiocin, nalidixic acid, or hydroxyurea were inoculated with strain CH34 (wild type) and shaken at 30°C. After 2 to 5 days, cells from the flask containing the highest concentration of the potential curing agent that just allowed growth were harvested, washed, diluted, and spread on agar plates containing Tris-gluconate medium and the respective metal salts. Plasmid-deficient mutants occurred at a frequency of 10^{-3} to 10^{-5} per mitomycin C-treated cell.

Enzyme activities. The activity of the cytoplasmic hydrogenase was determined photometrically by measuring NAD reduction in H_2-saturated phosphate buffer and that of the membrane-bound hydrogenase by manometric measurement of the hydrogen uptake rate under H_2 in the presence of methylene blue as described previously (34) or photometrically by measuring methylene blue reduction in H_2-saturated phosphate buffer. The method of Schink and Schlegel (32) was used, but glucose, glucose oxidase, and catalase were omitted. Anaerobic conditions were established by flushing the cuvette closed by a rubber stopper for 10 min with oxygen-free hydrogen. The activity of ribulosebisphosphate carboxylase were assayed radiometrically (8).

Matings. Donor and recipient strains were grown at 30°C in nutrient broth, and agar mating occurred as described by Lejeune et al. (22). The selective agar media contained Tris medium supplemented with 0.2% (wt/vol) gluconate and the following MICs of metals salts: 0.6 mM $NiCl_2$, 0.6 mM $CoCl_2$, 1.25 mM $ZnSO_4$, and 0.8 mM $CdCl_2$. Metal resistance of transconjugants was verified by using the following metal salt concentrations: 1 mM $NiCl_2$, 1 and 5 mM $CoCl_2$, 1 mM $ZnSO_4$, and 1 mM $CdCl_2$.

Isolation of plasmid DNA. Crude lysates of plasmid DNA were prepared as described by Kado and Liu (20).

Agarose gel electrophoresis. Electropherograms were prepared in 0.8% (wt/vol) agarose in 89 mM Tris-borate buffer (pH 8.5) plus 2.5 mM disodium EDTA (horizontal slab gel). Electrophoresis occurred at 150 V for 6 h. DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

RESULTS

Characterization of lithoautotrophic and heterotrophic growth. Strain CH34 is a gram-negative, motile, degenerately peritrichously flagellated rod. It is able to grow autotrophically in a mineral medium under a gas mixture con-

taining H_2, O_2, and CO_2 at various ratios; oxygen is tolerated up to 40% (vol/vol). It is a mesophilic bacterium able to grow autotrophically and heterotrophically up to 37°C, but not at 41°C. Under autotrophic conditions at 30°C the doubling time is 4 h. It does not grow on carbon monoxide, methane and methylamine.

Heterotrophic growth is supported by a large variety of organic compounds such as gluconate, succinate, malate, pimelate, azelate, suberate, sebacate, lactate, pyruvate, acetate, hexanoate, heptanoate, pelargonate, benzoate, 4-hydroxybenzoate, formate, glycerol, *n*-butanol, *n*-propanol, phenol, histidine, threonine, tryptophan, proline, and glycine, but not by fructose or other sugars. Rare mutants are able to grow on valine, leucine, and isoleucine (22); however, the selection of mutants able to grow on fructose, glucose, xylose, arabinose, and lactose was unsuccessful.

The rates of hydrogen oxidation by resting cells are significantly influenced by the presence of carbon dioxide. With autotrophically grown washed cells the gas uptake rate measured manometrically under H_2, O_2, and CO_2 is 3.54-fold higher than the rate measured under H_2 and O_2. This effect, described by Bartha (6) when studying *A. eutrophus* strain H16 and related strains, has so far only been encountered in species of hydrogen bacteria that contain both a cytoplasmic hydrogenase and a membrane-bound hydrogenase.

Activities of hydrogenases and ribulosebisphosphate carboxylase. Hydrogenases were present in autotrophically and heterotrophically grown cells. The total activity measured with whole cells manometrically under hydrogen with methylene blue as electron acceptor amounted to the following (micromoles of H_2 per minute and milligram of protein): autotrophically grown cells, 1.7; succinate-grown cells, 0.3; pyruvate-grown cells, 0.9; gluconate-grown cells, 1.0; glycerol-grown cells, 0.9; gluconate- and glycerol-grown cells, 1.5. Crude extracts obtained from autotrophically grown cells by sonication and centrifugation exhibited fairly high NAD-reducing hydrogenase activity; 1.02 μmol of NAD was reduced per min and mg of protein when measured photometrically at room temperature. The same extract reduced methylene blue at a rate of 2.2 μmol of H_2 per min and mg of protein when measured manometrically at 30°C. The membrane fraction alone did not reduce NAD, but reduced methylene blue at a rate of 0.72 μmol of H_2 per min and mg of protein at 30°C. In Table 2, the specific activities of the soluble and membrane-bound hydrogenases and ribulosebisphosphate carboxylase of strain CH34 grown on various substrates are listed. These experiments demonstrated the presence of both a cytoplasmic NAD-reducing hydrogenase and a membrane-bound hydrogenase in strain CH34. Furthermore, they showed that the formation of the hydrogenases and ribulosebisphosphate carboxylase is not dependent on the presence of hydrogen during growth. These enzymes are controlled by derepression rather than by induction. With respect to the hydrogenase pattern this strain does not significantly deviate from *A. eutrophus* H16 and other strains (N9A, B19) studied so far.

TABLE 2. Growth rates and activities of some key enzymes of autotrophic metabolism of *A. eutrophus* CH34 grown on various substrates[a]

Substrate	μ (1/h)	Sp act of enzymes (U/g of protein)		
		Soluble hydrogenase	Membrane-bound hydrogenase	Ribulosebisphosphate carboxylase
H_2 plus CO_2	0.12	1,230	747	806
Gluconate	0.20	199	896	11.5
Benzoate	0.39	46	87	0.3
Histidine	0.27	3	0	15.9
Pyruvate	0.30	6	11	0.8
Succinate	0.32	23	1	4.0
Ethanol	0.02	37	1,020	9.1
Acetate	0.26	41	1	1.8
Glycerol	0.02	228	316	15.8
Formate	0.04	586	246	173

[a] Autotrophic conditions for growth: 80% H_2O, 10% O_2, 10% CO_2.

TABLE 3. MICs of nickel, cobalt, zinc, and cadmium ions for various strains, mutants, and transconjugants of *A. eutrophus*

Phenotype	MIC (mM)	Representative clones
Nic^+	2.5	CH34, AE126, transconjugants carrying pMOL28
Nic^-	0.6	AE104, AE128, H16
Cob^+A	5.0	AE126, transconjugants carrying pMOL28
Cob^+B	20.0	CH34, AE128, transconjugants carrying pMOL30
Cob^-	0.07	AE104, clones lacking both plasmids
Zin^+	12.0	CH34, AE128, transconjugants carrying pMOL30
Zin^-	0.6	AE104, AE126
Cad^+	2.5	CH34, AE128, transconjugants carrying pMOL30
Cad^-	0.6	AE104, AE126

Growth inhibition by Ni^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} ions. Examination of growth of various strains on different media at varied concentrations of Ni^{2+}, Co^{2+}, Cd^{2+}, and Zn^{2+} revealed strong effects of medium composition on the inhibitory effect of the metals. On the usual mineral medium used for autotrophic growth of *A. eutrophus* H16 (34), cadmium and zinc did not inhibit colony growth even at a 30 mM. Apparently, the high phosphate content interferred with the metal effect, partially by precipitation. Thus Tris medium was used as the minimal medium. Tris buffer was not utilized as a nitrogen or carbon source, did not retard growth, did not result in chelating or precipitating the metals tested at growth-inhibitory ion concentrations, and gave the lowest MIC of heavy metals for a same strain, especially when compared with rich media. Consequently, Tris medium was used to differentiate between strains and mutants that are resistant or sensitive to the metal ions. The MIC values for metals are reported in Table 3.

Isolation of mutants sensitive to heavy metals. Various agents were employed to eliminate one or the other or both of the previously identified plasmids of 220 and 165 kb (17) from the cells. Mitomycin C treatment (4 μg/ml, 5 days) of strain CH34 resulted in the isolation of strain AE128, which lacks the small plasmid. After novobiocin (500 μg/ml, 3 days) treatment of AE128, mutant AE104 was isolated, which lacks both plasmids and has lost resistance to nickel, cobalt, zinc, and cadmium. Mitomycin C treatment (5 μg/ml, 2 days) of the wild-type CH34 resulted in the isolation of mutant AE126, which has lost the large plasmid and which shows a decreased resistance to cobalt, cadmium, and zinc (phenotype Ccz^-), but retains the resistance to nickel.

Furthermore, as the plasmids are not required for expression of hydrogenases and for autotrophic growth of strain CH34 and are mainly involved in heavy metal resistance, the following new designations were given: pMOL28 (formerly pHG13a) (17) with 163 kb and pMOL30 (formerly pHG13b) (17) with 238 kb. These values are derived from recent

TABLE 4. Transfer of resistance markers in intrastrain matings with the plasmid-free mutant as a recipient[a]

Donor	Plasmids	Recipient	No. of transconjugants per donor cell selected for resistance to:				
			Cadmium	Cobalt	Nickel	Zinc	Tetracycline and ampicillin
		AE104	$<10^{-10}$	10^{-9}	$<5 \times 10^{-10}$	$<10^{-10}$	$<10^{-10}$
AE81	pMOL28, pMOL30	AE104	10^{-8}	2×10^{-8}	5×10^{-8}	2.5×10^{-8}	
AE136	RP4, pMOL28, pMOL30	AE104	1.5×10^{-3}	5.3×10^{-3}	4×10^{-3}	1.5×10^{-3}	4 to 20
AE138	pUZ8, pMOL28, pMOL30	AE104	10^{-5}	1.2×10^{-5}	10^{-5}	10^{-5}	3
AE176	pMOL28	AE110		2×10^{-7}	2×10^{-7}		
AE194	pMOL30	AE110		1.5×10^{-7}		1.5×10^{-7}	

[a] The results are representative of four experiments for the mating AE104 × AE81 and of three experiments for the matings AE104 × AE136, AE110 × AE176, and AE110 × AE194. Viable counts indicate an excess of recipient CFU on the donor CFU by a factor 4 to 20. In the matings AE104 × AE136 and AE104 × AE138, RP4 or pUZ8 was transferred at a frequency near 100%. AE110 is a nalidixic acid-resistant derivative of AE104.

contour length measurements (W. Johannssen, personal communication) and differ slightly from values published previously (17). Thus, strain CH34 and its derivatives AE128, AE126, and AE104 contain two plasmids, the large plasmid pMOL30, the small plasmid pMOL28, and no plasmid, respectively. Electropherograms of these strains are shown in Fig. 1. The degrees of resistance of these four strains are described below. None of the mutants has lost the ability to grow autotrophically. This indicates that the plasmids pMOL28 and pMOL30 are not required for hydrogen oxidation or carbon dioxide fixation.

Degree of metal resistance of CH34 mutant derivatives. Overnight cultures of the wild type and the mutant derivatives were diluted and spread on Tris-mineral-gluconate plates (or TY plates for mercury) containing the metal salts at concentrations from 0.15 to 20 mM. Table 3 summarizes the MICs and the phenotypes of representative clones. The sensitivities of the mutants to nickel indicate that nickel resistance is correlated with pMOL28. In cobalt resistance both plasmids appear to be involved. The wild-type strain and AE128 exhibit the highest resistance to cobalt, whereas mutant AE126 exhibits intermediate cobalt resistance. The plasmid-free strain is dramatically sensitive to cobalt. This indicated that with respect to cobalt resistance an effect is exerted by both plasmids (Cob^+A and Cob^+B). However, both plasmids determine cobalt resistance to different levels; the presence of pMOL30 in CH34 and AE128 allowed colony growth up to 20 mM Co^{2+}, whereas pMOL28 in strain AE126 did not allow growth at higher concentrations than 1.25 mM Co^{2+}. The resistance to zinc and cadmium is well correlated with the presence of the large plasmid pMOL30. MIC of mercury is of 0.25 mM in the plasmid-free strain and around 0.7 mM in the plasmid-bearing strains.

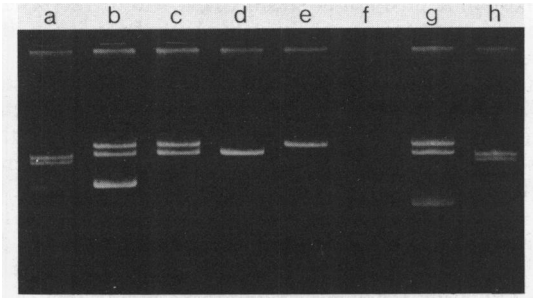

FIG. 1. Agarose gel electrophoresis of plasmid DNA of *A. eutrophus* strain CH34 and its derivatives as well as from *R. meliloti* MVII/1. Lanes: a and h, four different plasmids of *R. meliloti* MVII/1 listed from the top to the bottom were used as size standards, pRMVII/1a (157 kb) pRMVII/1b (136 kb), pRMVII/1c (82 kb), pRMVII/1d (56 kb); b, *A. eutrophus* CH34 containing the native plasmids pMOL30 (238 kb) and pMOL28 (163 kb) and plasmid pJP4 (77 kb); c, strain CH34 (wild type); d, mutant AE126; e, mutant AE128; f, mutant AE104; g, strain AE136 harboring plasmid RP4. Lysates were prepared as described previously (28).

Conjugational transfer of plasmids pMOL28 and pMOL30. The plasmid-free mutant AE104 of *A. eutrophus* CH34 can function as a recipient for pMOL28 and pMOL30 in intrastrain crosses. The matings were made between the plasmid-free strain AE104 and strain AE81, and selection occurred for Nic^+, Cob^+A, or Zin^+ clones; counterselection was performed by the omission of the three amino acids required by the donor strain. The transfer of the resistance markers occurred at very low frequencies (up to 10^{-8} per donor cell) (Table 4). IncP1 plasmids such as RP4 and pUZ8 as helper plasmids (24) were found to stimulate the transfer of pMOL28 and pMOL30 (Table 4). Similar results were obtained with plasmids pJP4 (10) and pULB113 (22) (data not shown).

Phenotype analysis of transconjugants. Transconjugants obtained in the matings AE104 × AE81 (mediated by pMOL28/pMOL30) and AE104 × AE136 (mediated by RP4) were analyzed for the presence of unselected markers (Table 5). The majority of transconjugants resulting from unassisted matings (without helper plasmid) of AE104 × AE81 had received all resistance markers. Only 12 of 42 transconjugants had received solely the Nic and CobA markers. Phenotypic analysis of the transconjugants obtained by IncP1-mediated transfer showed clearly that cotransfer of markers had occurred within two groups, namely, CobB, Zin, and Cad and Nic and CobA. The conjugational transfer mediated by the helper plasmid had resulted in a high number of transconjugants that had received only one of both linkage groups.

Matings with donors containing solely pMOL28 or pMOL30. Unassisted matings were performed with donors that contained only one plasmid, i.e., AE176 containing pMOL28 and AE194 containing pMOL30; both mutants were made lysine auxotrophic. The data (Table 4) indicate that each of both plasmids was transferred from these donors at a slightly higher frequency (about 2×10^{-7} per donor) than from AE81, which contains both plasmids. The experiments confirm that pMOL28 and pMOL30 are self-transmissible plasmids. They seem to mutually inhibit their unassisted transfer.

Physical analysis of plasmids of transconjugants. The plasmid bands of transconjugants obtained at very low frequencies in the unassisted mating AE104 × AE81 were analyzed by agarose gel electrophoresis, which was performed to relate the transferred phenotype to one of the two plasmids carried by *A. eutrophus* CH34 (Fig. 1). All trans-

TABLE 5. Phenotype analysis of transconjugants

Mating	Transfer mediated by:	Selection for phenotype	No. of clones tested	No. of clones representing linkage groups		
				(Cad^+, Cob^+B, Zin^+) (Nic^+)	(Cad^+, Cob^+B, Zin^+) (Nic^-)	(Nic^+, Cob^+A) (Cad^-, Cob^-B, Zin^-)
AE104 × AE81	pMOL28/pMOL30	Nic^+	42	30		12
		Cob^+	10	10	0	0
		Cad^+	6	6	0	
		Zin^+	12	11	1	0
AE104 × AE136	RP4	Nic^+	100	0		100
		Cob^+	75	0	17	58
		Cad^+	50	0	50	
		Zin^+	50	0	50	
AE104 × AE138	pUZ8	Nic^+	50	0		50
		Cob^+	75	0	48	27
		Cad^+	25	0	25	
		Zin^+	75	0	75	

conjugants, which were of the wild-type phenotype, harbored two plasmids indistinguishable from the plasmids of strain CH34 (data not shown). Transconjugants of the phenotype Nic^+ Cob^+A Zin^- Cob^-B Cad^- have only one plasmid of the same size as pMOL28 (five transconjugants tested). One transconjugant, AE150, selected for resistance to zinc, carried only one plasmid, pMOL53 (about 105 kb), apparently a derivative of pMOL30 (data not shown). The unassisted transfer seems thus to involve both plasmids together and to result sometimes in rearrangement.

The plasmid bands of transconjugants obtained in the mating AE104 × AE136, thus assisted by RP4, were also analyzed (data not shown). AE158 is a transconjugant selected for nickel resistance; AE161 and AE162 were selected for zinc resistance. All three of them show bands corresponding to plasmids pMOL28 (AE158) and pMOL30 (AE161 and AE162). In addition, a plasmid somewhat heavier than RP4 is present. Insertions of around 6 to 9 kb in RP4 and in pUZ8 were systematically observed after mobilization of pMOL30 or pMOL28 (24). Some led to inactivation of the gene encoding resistance to tetracycline (24). All of them confer resistance to mercury compounds when they are transferred to *Escherichia coli* recipients (data not shown). This was confirmed by Davidson and Summers (personal communication) with transfer experiments with AE136 as a donor. pUZ8 is an IncP1 plasmid without known transposable elements (37). This suggests that the RP4- or pUZ8-assisted transfer of plasmids pMOL28 and pMOL30 occurs via a cointegrate involving a transposable element present in strain CH34 and recovered in RP4 or pUZ8 after the resolution of the cointegrate in the recipient cell (24). The plasmid formula of AE159 (Nic^+ transconjugant) probably reflects the formation of such a cointegrate between RP4 and pMOL28.

The results obtained in the matings with and without helper plasmids show clearly the separation of the phenotype Nic^+ Cob^+A assigned to pMOL28 and of Cad^+ Cob^+B Zin^+ assigned to pMOL30 and thus confirm the conclusions based on the analysis of the cured mutants AE126 and AE128.

DISCUSSION

A. eutrophus strain CH34 shares with other strains (H16, N9A, G27, G29, B19, type strain) of the species the presence of two hydrogenases. However, besides the resistance to heavy metal there are at least two major characters that differentiate CH34 from other strains of this species examined so far: one concerns its inability to use fructose as a carbon source, and the other concerns the absence of a conjugative megaplasmid similar to pHG1, which specifies the genes for the hydrogenases in strain H16 (other differences were reported by Steinbüchel et al. [35]). Instead, strain *A. eutrophus* CH34 carries two plasmids specifying resistances against cobalt, nickel, cadmium, mercury, and zinc. These resistances are located on the two plasmids pMOL28 and pMOL30. pMOL30 carries resistances against zinc, mercury, and cadmium as well as high level cobalt resistance called *cobB*. The smaller plasmid pMOL28 carries resistance against nickel and mercury and a lower-level cobalt resistance called *cobA*. These findings were proved by (i) determining the resistance patterns of strains AE104 (without plasmids), AE128 (with pMOL30), AE126 (with pMOL28), and the wild-type CH34 (with both plasmids); and (ii) linkage group analysis of transconjugants followed by physical plasmid analysis.

No resistance to the normal antibiotics was found to be associated with pMOL28 and pMOL30. Since these plasmids are not required for hydrogenase formation in strain CH34, we conclude that the genetic information for the hydrogenases is located on the chromosome in strain CH34. The taxonomic position of strain CH34 will be reconsidered as soon as more strains of this type have been isolated and studied.

Strain CH34 has a high susceptibility to IncP1 plasmids. Plasmid pJP4, which codes for 2,4-dichlorophenoxyacetic acid degradation (10), is transferred to strain CH34 with the highest frequency among the *A. eutrophus* strains examined (15). As the IncP1 plasmids are very stable in strain CH34, as are the native plasmids pMOL28 and pMOL30, the latter plasmids do not belong to this compatibility group. Both plasmids proved to be self-transmissible in intrastrain matings. Comparing the mutants harboring solely one of both plasmids with the wild type, the transfer frequency was at least 1 order of magnitude higher. Thus, pMOL28 and pMOL30 apparently mutually inhibit their transfer.

The conjugational transfer of both plasmids is enhanced by IncP1 helper plasmids. This may be the result of the use of *mob* functions of the CH34 plasmids by the *tra* genes of the helper plasmid or of integration of CH34 native plasmids into IncP1 plasmids; this second possibility is strongly supported by the rearrangements observed in RP4 and pUZ8

in the transconjugants that have inherited pMOL28 or pMOL30 (24).

Thus, *A. eutrophus* CH34 is the first gram-negative bacterium showing plasmid-bound resistance to cadmium and zinc. These resistances have only been observed in the gram-positive *Staphylococcus aureus* (11, 29; for a review, see reference 13).

In *S. aureus* cadmium resistance is linked with zinc resistance (28). Each resistance is coded by two genes differing with respect to localization, level of resistance, and function; *cadA* determines a high-level resistance mediated by an efflux mechanism (36), and *cadB* codes for a lower-level resistance and has been found only on staphylococcal pII plasmids (13). The physiological function has not been elucidated. *cadB* possibly codes for a binding protein similar to eucaryotic metallothioneins (21). Similar proteins have been found in a cyanobacterium (30) and in *Pseudomonas putida* (19). Because of the *S. aureus* example and the chemical relatedness of zinc and cadmium, a physiological linkage of both resistances is possible in strain CH34 too. However, in CH34, resistance to zinc is more striking than resistance to cadmium (Table 3) and seems to be the obvious response of the bacterium to the environmental constraint, since CH34 was isolated from a decantation tank highly contaminated by Zn^{2+} (26). Resistance to mercury is correlated to both pMOL28 and pMOL30 and seems to be linked to a transposon able to move on plasmid RP4 (24). Plasmid-bound resistance to mercury was extensively reviewed and leads essentially to detoxication by reduction of Hg^{2+} to Hg^0 (31). Cobalt and nickel are chemically related; both are transported into cell by the magnesium system (1, 5, 16, 23, 27, 38), and both act toxically in having iron metabolism as their target in the case of fungi (2, 18). So *cobA* and the nickel resistance might be linked. Some of these questions will be answered by further genetic and physiological studies.

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LITERATURE CITED

1. **Abelson, P. H., and E. Aldous.** 1950. Ion antagonism in microorganisms: interference of normal magnesium metabolism by nickel, cobalt, cadmium, zinc and manganese. J. Bacteriol. **60**:401–413.
2. **Adiga, P. R., K. Sivarama Sastry, V. Venkatasubramanyam, and P. S. Sarma.** 1961. Interrelationships in trace-element metabolism in *Aspergillus niger*. Biochem. J. **81**:545–550.
3. **Anderson, K., R. C. Tait, and W. R. King.** 1981. Plasmids required for utilization of molecular hydrogen by *Alcaligenes eutrophus*. Arch. Microbiol. **129**:384–390.
4. **Aragno, M., and H. G. Schlegel.** 1977. *Alcaligenes ruhlandii* (Packer and Vishniac) comb. nov., a peritrichous hydrogen bacterium previously assigned to *Pseudomonas*. Int. J. Syst. Bacteriol. **27**:279–281.
5. **Babich, H., and G. Stotzky.** 1983. Toxicity of nickel to microbes: environmental aspects. Adv. Appl. Microbiol. **29**: 195–265.
6. **Bartha, R.** 1962. Physiologische Untersuchungen über den chemolithotrophen Stoffwechsel neu isolierter Hydrogenomonas Stämme. Arch. Mikrobiol. **41**:313–350.
7. **Biebl, H., and N. Pfennig.** 1981. Isolation of members of the family Rhodospirillaceae, p. 267–273. *In* M. P. Starr, J. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes, a handbook on habitats, isolation and identification of bacteria, vol. 1. Springer-Verlag, Berlin.
8. **Bowien, B., F. Mayer, G. A. Codd, and H. G. Schlegel.** 1976. Purification of some properties and quaternary structure of the D-ribulose 1,5diphosphate carboxylase of *Alcaligenes eutrophus*. Arch. Microbiol. **110**:157–166.
9. **Bowien, B., and H. G. Schlegel.** 1981. Physiology and biochemistry of aerobic hydrogen oxidizing bacteria. Annu. Rev. Microbiol. **35**:405–452.
10. **Don, R. A., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145**:681–686.
11. **Dyke, K. H. G., M. T. Parker, and T. Richmond.** 1970. Penicillinase production and metal ion resistance in *Staphylococcus aureus* cultures isolated from hospital patients. J. Med. Microbiol. **3**:125–136.
12. **Echols, H., A. Garen, S. Garen, and A. Torriani.** 1961. Genetic control of repression of alkaline phosphatase in *E. coli*. J. Mol. Biol. **3**:425–438.
13. **Foster, T. J.** 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47**:361–409.
14. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occuring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147**:198–205.
15. **Friedrich, B., M. Meyer, and H. G. Schlegel.** 1983. Transfer and expression of the herbicide degrading plasmid pJP4 in aerobic autotrophic bacteria. Arch. Microbiol. **134**:92–97.
16. **Fuhrmann, G. F., and A. Rothstein.** 1968. The transport of Zn^{2+}, Co^{2+} and Ni^{2+} into yeast cells. Biochim. Biophys. Acta **163**:325–330.
17. **Gerstenberg, C., B. Friedrich, and H. G. Schlegel.** 1982. Physical evidence for plasmids in autotrophic, especially hydrogen-oxidizing bacteria. Arch. Microbiol. **133**:90–96.
18. **Healy, W. B., S. Cheng, and W. D. McElroy.** 1955. Metal toxicity and iron deficiency effects on enzymes in *Neurospora*. Arch. Biochem. Biophys. **54**:206–214.
19. **Higham, D. P., P. Sadler, and M. D. Scawen.** 1984. Cadmium-resistant *Pseudomonas putida* synthesizes novel cadmium proteins. Science **225**:1043–1045.
20. **Kado, C. I., and S. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
21. **Lerch, K.** 1980. Copper metallothionein, a copper-binding protein from *Neurospora crassa*. Nature (London) **284**:368–370.
22. **Lejeune, P., M. Mergeay, F. Van Gijsegem, M. Faelen, J. Gerits, and A. Toussaint.** 1983. Chromosome transfer and R-prime formation mediated by plasmid pULB113 (RP4::miniMu) in *Alcaligenes eutrophus* CH34 and *Pseudomonas fluorescens* 6.2. J. Bacteriol. **155**:1015–1026.
23. **Maruthi Mohan, P., and K. Sivarama-Sastry.** 1983. Interrelationships in trace-element metabolism in metal toxicities in nickel-resistant strains of *Neurospora crassa*. Biochem. J. **212**:205–210.
24. **Mergeay, M., M. Faelen, and J. Gerits.** 1984. IncP1 plasmids stimulate the transfer of large plasmids controlling multiple resistance to heavy metals in *Alcaligenes eutrophus*. Arch. Int. Physiol. Biochem. **92**:45–46.
25. **Mergeay, M., J. Gerits, and C. Houba.** 1978. Facteur transmissible de la resistance au cobalt chez un Pseudomonas du type hydrogenomonas. C. R. Soc. Biol. **172**:575–579.
26. **Mergeay, M., C. Houba, and J. Gerits.** 1978. Extrachromosomal inheritance controlling resistance to cadmium, cobalt, and zinc ions: evidence from curing in a Pseudomonas. Arch. Int. Physiol. Biochim. **86**:440–441.
27. **Nelson, D. L., and E. P. Kennedy.** 1971. Magnesium transport in *Escherichia coli*. Inhibition by cobaltous ion. J. Biol. Chem. **246**:3042–3049.
28. **Novick, R. P., E. Murphy, T. J. Cryczan, E. Baron, and I. Edelman.** 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid **2**:109–129.
29. **Novick, R. P., and C. Roth.** 1968. Plasmid-linked resistance to

inorganic salts in *Staphylococcus aureus*. J. Bacteriol. **95**:1335–1342.
30. **Olafson, R. W., K. Abel, and R. S. Sim.** 1979. Prokaryotic metallothionein: preliminary characterization of a blue green algae heavy metal-binding protein. Biochem. Biophys. Res. Commun. **89**:36–43.
31. **Robinson, J. B., and O. A. Tuovinen.** 1984. Mechanisms of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical, and genetic analyses. Microbiol. Rev. **48**:95–124.
32. **Schink, B., and H. G. Schlegel.** 1979. The membrane-bound hydrogenase of *Alcaligenes eutrophus*. I. Solubilisation, purification, and biochemical properties. Biochim. Biophys. Acta **597**:315–324.
33. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Sumbersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstum physiologische Untersuchungen. Arch. Mikrobiol. **38**:209–222.
34. **Schneider, K., and H. G. Schlegel.** 1977. Localization and stability of hydrogenase from aerobic hydrogen bacteria. Arch. Microbiol. **112**:229–238.
35. **Steinbüchel, A., M. Kuhn, M. Niedrig, and H. G. Schlegel.** 1983. Fermentation enzymes in strictly aerobic bacteria: comparative studies on strains of the genus *Alcaligenes* and on *Nocardia opaca* and *Xanthobacter autotrophicus*. J. Gen. Microbiol. **129**:2825–2835.
36. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **147**:313–319.
37. **Villaroel, R., R. W. Hedges, R. Maenhaut, J. Leemans, G. Engler, M. Van Montagu, and J. Schell.** 1983. Heteroduplex analysis of P-plasmid evolution: the role of insertion and deletion of transposable elements. Mol. Gen. Genet. **189**:390–399.
38. **Webb, M.** 1970. Interrelationship between the utilization of magnesium and the uptake of other bivalent cations by bacteria. Biochim. Biophys. Acta **222**:428–439.